REMARKS

Rejections under 35 U.S.C. 112

Claims 1-4 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claims are said to contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner states that applicants have provided support only for the preparation of a human plasminogen fragment.

This rejection is respectfully traversed. The claims have now been amended to recite that the fragment is a human plasminogen fragment.

Claim 1 is rejected under 35 U.S.C. 112, first paragraph, because the specification is said to be enabling only for a composition for inhibiting lung tumor metastasis and lung tumor growth.

This rejection is respectfully traversed. The claims have now been amended to recite that the composition is for inhibiting lung tumor metastasis and lung tumor growth.

Clams 1-4 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The recitation "binds less intensely" is said to be vague and indefinite.

This rejection is respectfully traversed. Claim 1 has now been amended to recite that it binds to heparin at pH lower than neutral pH but does not bind to heparin at neutral or higher pH, under physiological ionic conditions.

Art Rejections

Claims 1-4 are rejected under 35 U.S.C. 102(b) as being anticipated by Reich et al., U.S. Patent No. 5,288,489. The Examiner concedes that Reich et al. do not specifically recite the molecular weight, lack of glycosylation, heparin binding activity and inhibiting tumor metastasis and tumor growth, the Examiner alleges that these limitations would be inherent qualities of the recovered compound.

Claims 1-4 are also rejected under 35 U.S.C. 102(b) as being anticipated by Davidson, U.S. Patent No. 5,801,146. This rejection is respectfully traversed.

Submitted herewith are copies of two declarations of the inventor, Wataru MORIKAWA, that had been submitted in the parent application, Serial No. 09/269,720. It can clearly be seen from these declarations that the fragment of the present

invention has the ability to inhibit lung tumor metastasis and lung tumor growth, but has substantially no activity in inhibiting growth of endothelial cells of blood vessels, as is the activity claimed for the fragments of Reich et al. and Davidson.

The fragment of the present invention (*i.e.*, Lys-Lysine Binding Site I, hereinafter referred to as "Lys-LBS-I") is characteristic in that it (1) has a molecular weight of 38 kDa; (2) it is not glycosylated; (3) it binds intensely to heparin wherein it binds less intensely to heparin or heparin-like substances under physiological (isotonic) conditions, *i.e.*, at around neutral pH, but binds intensely to heparin under non-physiological conditions, *e.g.*, at a lower environmental pH such as within the tumor region; and (4) it has the ability to inhibit lung tumor metastasis and lung tumor growth, but has substantially no activity in inhibiting growth of endothelial cells of blood vessels.

The fragment of the present invention is prepared by (a) preparing Lys-Plasminogen (hereinafter also referred to as "Lys-[;g") with N-terminal lysine-78 from human plasminogen either through addition of plasmin to a solution of human plasminogen or through incubation of human plasminogen in the presence of tranexamic acid to autolysis; (b) treating the Lys-plg obtained in step (a) with elastase to produce

fractions of the fragment comprising Kringle 1 to Kringle 3; (c) identifying the fragment of Kringle 1 to Kringle 3 which binds to heparin; and (d) isolating the fragment which binds to heparin.

In comparison with the plasminogen fragments disclosed in Davidson and Reich et al., the most distinctive features of the fragment claimed herein are that it is able to inhibit lung tumor metastasis and lung tumor growth, but it has substantially no activity for inhibiting growth of endothelial cells of blood vessels, unlike the fragments of Reich et al. and Davidson. This biological activity of the fragment claimed herein is based on its special high-order structure that can be established by Lys-LBS-I with N-terminal lysine-78 without glycosylation. Furthermore, it should be noted that such a special high-order structure is associated with an ability of the fragment to bind heparin intensely. Thus, the fragment of the present invention may be defined as "Lys-LBS-I with N-terminal lysine-78 without glycosylation that binds to heparin wherein a special high-order structure is established."

The relationship between the ability to bind to heparin (heparin affinity) and the difference at the N-terminal sequence (Glu-LBS-I or Lys-LBS-I, more specifically, N-terminal lysine-78, valine-79, tyrosine-80 or leucine-81),

and the relationship between an ability to bind heparin (heparin affinity) and the presence or absence of glycosylation, are demonstrated in Declaration I by Wataru MORIKAWA.

The "Glu-LBS-I" fraction was prepared by treating Glu-plasminogen with elastase, whereas the "Lys-LBS-I" fraction is obtained by treating plasminogen with plasmin to prepare a molecule with N-terminal lysine-78 (*i.e.*, Lys-plg), and treating Lys-plg with elastase. The Glu-LBS-I and the Lys-LBS-I fractions were divided into three groups depending upon the degree of heparin affinity, *i.e.*, fractions with high, medium and low heparin affinity. Each fraction was analyzed for its N-terminal amino acid residue and for the presence or absence of any glycosylation.

Declaration 1 demonstrates:

(a) that the fraction with high heparin affinity from Lys-LBS-I comprises predominantly Lys-LBS-I with N-terminal lysine-78, while Lys-LBS-I with N-terminal valine-79 or tyrosine-80 was present in a trace amount. Glu-LYS-I obtained from Glu-plasminogen, hereinafter also referred to as "Glu-plg", through direct elastase cleavage comprises predominantly Glu-LBS-I with N-terminal tyrosine-80 and a small amount with N-terminal leucine=81, irrespective of heparin activity. Glu-LBS-I with N-terminal lysine or valine-

79 was utterly undetectable in the fractions from Glu-LBS-I; and

(b) fractions with high heparin affinity from Lys-LBS-I or Glu-LBS-I comprise predominantly Lys-LBS-I or Glu-LBS-I without glycosylation, respectively, and it appeared that a degree of heparin affinity was conversely correlated with the extent of glycosylation.

Prior to contrasting the presently claimed invention with the disclosures of Reich et al. and Davidson, a discussion of the background of the present invention, plasminogen fragments obtained by enzymatic cleavage of plasminogen with elastase and a mechanisms of the phenomenon of vascularization.

Plasminogen Fragments

It is known that elastase cleavage of plasminogen provides Lysine Binding Site I, hereinafter referred to as "LBS-I", consisting of K1 to K3 Kringle domains, Lysine Binding Site (LBS-II) consisting of K4 domain, and Mini-plasminogen consisting of K5 domain and serine protease domain. With respect to LBS-I, it should be noted that this is not a single molecular species, but includes several different molecular species with different degrees of

glycosylation and different high-order structures to form subpopulations.

That is, LBS-I has two glycosylation sites, and thus there are three types of molecular species, namely, 45, 40, and 38 kDa, depending on the presence or absence of these two glycosylations. Furthermore, it is assumed that LBS-I is able to build up different high-order structures to exert different biological activities. For example, some species having a special high-order structure exhibit the activity of inhibiting growth of endothelial cells of blood vessels, whereas another species with different high-order structure does not have this activity but exhibits another activity, *e.g.*, the ability to inhibit sprouting of endothelial cells of blood vessels. Figure 1 attached hereto illustrates a schematic view of LBS-I molecular species.

It should be noted that there are at least two types of LBS-I, *i.e.*, Glu-LBS-I and Lys-LBS-I with N-terminal tyr-80 and N-terminal lys-78 derived from Glu-plg and Lys-plg, respectively. Although there is only a slight difference in amino acids at the N-terminal between Glu-LBS-I and Lys-LBS-I (only two additional amino acid residues in Lys-LBS-I at the N-terminal), the high-order structure of these two moieties is

significantly different, resulting in distinct biological activities.

Mechanism of Vascularization

Vascularization is the generation of new blood vessels from the existing blood vessels into a tissue or organ, and is one of the key mechanisms of proliferation of cancer cells. Vascularization is accomplished through multiple steps: (1) growth of endothelial cells of blood vessels; (2) degradation of the basement membrane of blood vessels; (3) sprouting of endothelial cells of blood vessels; and (4) canalization.

Blockage of any one of these four steps can lead to inhibition of vascularization, and hence to inhibition of proliferation of cancer cells. Thus, it is clear that inhibition of vascularization aimed at inhibiting tumor metastasis and tumor growth may be attained not only by inhibiting growth of endothelial blood vessels, as in step 1, but also by inhibiting any of the other steps.

For example, it has been reported that plasmin, the active form of plasminogen, and matrix metallo proteinases are involved in step 2, degradation of the basement membrane of blood vessels. This, matrix metallo proteinase inhibitor (MMP inhibitor) has been shown to inhibit vascularization and cancer cells' proliferation by inhibiting degradation of the

basement membrane of blood vessels, even though it does not inhibit growth of endothelial cells of blood vessels. MMP inhibitor has already been the subject of a clinical trial.

Reich et al. discloses fibrinolysis and fibrinogenolysis treatment with plasmin or its precursor plasminogen in fibrinolytically and fibrinogenolytically active forms. Reich et al. disclose Lys-plg and preparation thereof from naturally occurring molecular species Glu-plasminogen by the catalytic activity of plasmin (column 8, lines 47-52). Reich et al. also teach that mini-plasminogen is derived from either Glu- or Lys-plg by limited proteolysis, catalyzed by pancreatic elastase, whereby a fragment consisting of the proenzyme domain of plasminogen with a single attached Kringle is generated (column 8, lines 52-56).

The present invention, on the other hand, is directed to inhibiting lung tumor metastasis and lung tumor growth. That is, the target disease state or condition to be treated are quite different from those of Reich et al. Thus, Reich et al. do not anticipate the present invention.

As noted above, the high-order structure of Lys-LBS-I is quite significant for exerting the desired activity, that of inhibiting tumor metastasis and tumor growth according to the present invention. For such a high-order structure, a specific N-terminal starting from lysine-78 and the absence of

glycosylation are essential. In this regard, Reich et al. are silent about the presence or absence of glycosylation in plasminogen fragment as, as well as the consequent high-order structure. Accordingly, it is respectfully submitted that Reich et al. neither teach nor suggest the fragment of the present invention.

The Examiner asserts that lack of glycosylation and heparin binding activity would be inherent properties of the recovered compounds, particularly in light of the fact that the method of Reich et al. and the method of the present invention are the same. It is respectfully submitted that this is not the case, since lack of glycosylation and heparin binding activity are not inherent properties of the recovered compounds. As explained above, the recovered compounds include plural molecular species of Lys-LBS-I, depending on the presence and absence of glycosylation and high-order structure. Thus, for obtaining the Lys-LBS-I of the present invention, this must be screened for its ability to bind to heparin. Accordingly, Reich et al. never teach or suggest the Lys-LBS-I which would inherently exhibit a heparin binding activity and thus inhibition of lung tumor metastasis or lung tumor growth.

Davidson discloses an isolated Kringle 5 of mammalian plasminogen as a compound for treating angiogenic

diseases, and methods and compositions for using the Kringle 5. It is apparent that Davidson's Kringle 5 is different from the fragment comprising Kringle 1 and Kringle 3 of the present invention.

In the Background section, Davidson also discloses production of angiostatin by elastase digestion of Lys-plg, in a manner similar to Folkman et al., U.S. Patent No. 5,837,682, (column 2, lines 15-30), cited during prosecution of the parent application. This patent is discussed for the background of endothelial inhibitors.

Folkman et al. disclose endothelial inhibitors, called angiostatin, which reversibly inhibits proliferation of endothelial cells and methods of use thereof. The angiostatin disclosed by Folkman et al. has been shown to be capable of inhibiting the growth of endothelial cells *in vitro* (column 12, lines 35-36). Folkman defines "angiostatin fragment" as a protein derivative of angiostatin, or plasminogen, having an endothelial cell proliferation inhibiting activity (column 41, lines 18-20). Folkman et al. also disclose that lysine binding site I, or angiostatin, is a population of proteins that contain, the aggregate, at least the first three triple-loop structures (number 1 through 3) in the plasmin A-chain (Kringle 1+2+3) (column 33, lines 32-38).

Thus, **the molecular species disclosed in Folkman et al. is an endothelial inhibitor which has an endothelial cell proliferating activity that enables suppression of tumor metastasis.**

In contrast thereto, the Lys-LBS-I of the present invention has been found **not** to have an endothelial cell proliferation inhibiting activity, but rather the activity to inhibit lung tumor metastasis and lung tumor growth. The active moiety of the present invention is distinct from the "angiostatin" of Folkman et al.

As explained above, LBS-I is not a single molecular species, but includes several different molecular species with different degrees of glycosylation and different high-order structure to form subpopulations. Such different degrees of glycosylation and high-order structures in various molecular species give these molecular species different biological activities, *e.g.*, the presence and absence of an activity to inhibit growth of endothelial cells, or an activity to inhibit degradation of the basement membrane of blood vessels, etc. Thus, based upon the differences in their activity, the Lys-LBS-I of the present invention is well distinguished from the "angiostatin" as disclosed in Folkman et al. Even though both the Lys-LBS-I of the present invention and the "angiostatin" of Folkman et al. may lead to inhibition of vascularization

and hence to suppression of tumor metastasis, it is through inhibition of different steps in the vascularization mechanism. It is clear that since the active moieties of the present invention and of Folkman et al. act in different ways, they cannot be the same.

The present inventors have demonstrated that commercially available angiostatin (angiogenesis inhibitor K1-3, *Technoclone, Inc.*, Blood 1998), Glu-LBS-I, could effectively inhibit growth of endothelial cells of blood vessels, wherein the Lys-LBS-I of the present invention did not exhibit such activity even at a concentration 10- or 20-fold higher than that of the angiostatin. This was demonstrated in Declaration 2 of Wataru MORIKAWA, submitted in the parent application, a copy of which is submitted herewith.

Folkman et al. disclose that the fragments of K1-3, K1-4 and K4 were prepared by digesting Lys-Hpg (human Lys-plg) with elastase (column 44, lines 12-21) and purified (column 44, lines 58-67). However, Folkman et al. disclose at column 46, line 14, that the N-terminal amino acid sequence analysis of the purified fragments revealed that K1-3 had the N-terminal-YLSE, *i.e.*, N-terminal tyrosine-80.

In contrast thereto, the Lys-LBS-I of the present invention has predominantly N-terminal lysine-78 and a minor amount of MN-terminal valine-79. It has been found that when

Lys-plg is digested with elastase, molecular species with either N-terminal lysine-78 or valine-79 are obtained, with only a contaminating trace amount of molecular species with N-terminal tyrosine-80. As shown in Declaration 1, the fraction with high heparin affinity is comprised predominantly of Lys-LBS-I with N-terminal lysine-78, while Lys-LBS-I with N-terminal tyrosine-80 accounts for merely about 3% of high heparin affinity fraction.

That is, the purified fragment with N-terminal tyrosine-80 obtained in Folkman et al. is distinct from the Lys-LBS-I with N-terminal lysine-78 of the present invention, and does not have the property of binding to heparin.

Folkman et al. also disclose that the fragments of K1-3 and K1-4 prepared by digesting Lys-plg with elastase are purified using a **heparin**-Sepharose column (column 44, lines 58-67). However, it should be noted that this purification/separation is performed for separating K1-3 from K1-4 molecular species, but not for separating three different species of K1-3 (*i.e.*, LBS-I) which are 38, 40 and 45 kDa, from each other.

In view of the above, it is respectfully submitted that the claims are now in condition for allowance, and favorable action thereon is earnestly solicited.

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Wataru MORIKAWA et al.
Serial No. 09/269,720 Group Art Unit: 1642
Filed: April 6, 1999 Examiner: Jennifer Nichols
For: PLASMINOGEN FRAGMENT
HAVING ACTIVITY TO INHIBIT
TUMOR METASTASIS AND GROWTH...

DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231
U.S.A.

Sir:

I, Wataru MORIKAWA, a citizen of Japan residing 14-29, Kusunoki 7-chome, Kumamoto-shi, Kumamoto-ken, Japan, declare as follows:

1. I was graduated from Shimane University, Faculty of Agriculture, Department of Agricultural chemistry in 1983, finished the graduate school (master course) in said University in 1985.

2. Since 1985 up till the present, I have been in the employ of Juridical Foundation The Chemo-Sero-Therapeutic Research Institute. Since 1999 up till the present, I have been prosecuting the study of angiogenesis inhibitor at Kyushu University, Faculty of Medicine, Chair of First Biochemistry.

From April 1985 up till 1987, I had been engaged in the research work with respect to development of blood coagulation factor, from 1987 up till 1995, to development of blood coagulation factor and investigation of useful factors in blood (study on relationship between lipoproteins such as HDL or Lp(a) and arteriosclerosis), and from 1995 up till 1999, to investigation of angiogenesis inhibitor (angiostatin) at said Foundation.

3. For the past years, I had made several reports as listed below:

1) Acetylated low density lipoprotein reduces its ligand activity for the scavenger receptor after interaction

with reconstituted high density lipoprotein, J. Biol. Chem., 1994 Feb.18: 269(7): 5264-5269

2) Comparison of monoclonal and polyclonal enzyme-linked immunoabsorbent (ELISA) assays for serum Lp(a) and differences in reactivities to Lp(a) phenotypes, J. Clin. Lab. Anal., 1995: 9(3): 173-177

3) Measurement of Lp(a) with a two-step monoclonal competitive sandwich ELISA method, Clin. Biochem., 1995 June: 28(3): 269-275

4) Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits, Arterioscler. Thromb. Vasc. Biol., 1995 Nov.15 (11): 1882-1888

5) Reconstituted high density lipoprotein reduces the capacity of oxidatively modified low density lipoprotein to accumulate cholesterol esters in mouse peritoneal macrophages, Atherosclerosis, 1996 Jan.26: 119(2): 191-202

6) Lipoprotein (a) induces cell growth in rat peritoneal macrophages through inhibition of transforming growth factor-beta activation, Atherosclerosis, 1996 Aug. 23:125(1): 15-26

7) The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13, J. Immunol., 2000 Sep. 1: 165(5): 2818-2823

4. I am a member of The Japanese Cancer Society, The Japanese Society for Pediatrics, The Japanese Society for Arteriosclerosis and The Japanese Society for Clinical Pathology.

5. I am one of the inventors in the instant U.S. application and familiar with the subject matter thereof.

6. I have read Reich et al. (USP 5,288,489), Folkman et al. (USP 5,837,682) and Davidson (USP 5,801,146), and am familiar with the subject matter thereof.

7. Under my direction, the following comparative experiments have been done.

Materials and Methods

(1) Lys-Lysine Binding Site I

Lys-Lysine Binding Site I (hereinafter referred to as "Lys-LBS-I") was prepared as described in Example 4 of the instant application.

(2) Glu-Lysine Binding Site I

"Angiogenesis-Inhibitor Kringle 1-3" (Cat. No. 41022; Technoclone (TC), Müllnergasse 23, A-1090 Vienna, Austria) was used for Glu-Lysine Binding Site I (hereinafter referred to as "Glu-LBS-I").

"Angiogenesis-Inhibitor Kringle 1-3" was prepared from human Glu-plasminogen (hereinafter referred to as "Glu-plg.")(TC Cat. No. 41004) by elastase digestion followed by affinity chromatography on lysine sepharose and further purification [(1)].

[(1)]: Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E., Magnussen, S. (1978) in Progress in Chemical Fibrinolysis and Thrombolysis (Davidson, J.F., Rowan, R.M., Samama, M.M., and Desnoyers, P.C., eds.) Vol. 3, pp. 191-209, Raven Press, New York

(3) Kringle 1-4 fragments

Glu-plg. was digested with cathepsin D to prepare Kringle 1-4 fragment (PAN4).

Glu-plg. was also digested with plasmin to prepare Kringle 1-4 fragment (GAN).

(4) HOME cell proliferation assay 1

Human microvascular endothelial (HOME) cells were plated on 24-well culture plate in 1 ml of M199/10% FCS medium at 12,500 cells/well. The plate was incubated at 37°C overnight and then the medium was exchanged with 250 µl M199 serum free medium and test samples were added thereto with control being added no test samples. After incubation for 3 hours, 250 µl of M199 medium containing 10% FCS was added to the plate. After incubation for 72 hours, the cells were counted with Coulter counter.

(5) HOME cell proliferation assay 2

HOME cells were plated on 24-well culture plate in 1 ml of M199/10% FCS medium at 12,500 cells/well. The plate was

incubated at 37°C overnight and then the medium was exchanged with 250 µl M199 serum free medium and test samples at various concentrations (0 to 40 µg/ml) were added thereto. After incubation for 3 hours, 250 µl of M199 medium containing 10% FCS was added to the plate. After incubation for 72 hours, the cells were counted with Coulter counter.

Results

The results are shown in the attached Figures 1 and 2.

Figure 1 demonstrates that Lys-LBS-I did not inhibit proliferation of HOME cells at both concentrations of 80 and 160 µg/ml whereas Glu-LBS-I (TC K1-3) and Kringle 1-4 fragments (PAN 4 and GAN) inhibited proliferation of HOME cells at 80 µg/ml.

Figure 2 demonstrates that Lys-LBS-I did not inhibit proliferation of HOME cells at concentrations up to 40 µg/ml whereas Glu-LBS-I (TC K1-3) and Kringle 1-4 fragment (PAN 4) inhibited proliferation of HOME cells at around 5 to 10 µg/ml.

The above results clearly demonstrate that Glu-LBS-I obtained by elastase cleavage of Glu-plg. inhibits proliferation of endothelial cells of blood vessels whereas Lys-LBS-I of the present invention does not inhibit the proliferation even at a concentration 10- to 20-fold higher than that of Glu-LBS-I.

8. It is my opinion based on my knowledge and experience in this field that Glu-LBS-I inhibits proliferation of endothelial cells of blood vessels whereas Lys-LBS-I of the present invention does not inhibit the proliferation even at a concentration much higher than that of Glu-LBS-I. Thus, Lys-LBS-I of the present invention is well distinguished from Glu-LBS-I in that the latter inhibits proliferation of endothelial cells of blood vessels whereas the former not.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-mentioned application or any patent issuing thereon.

This day of September, 2000.

Wataru MORIKAWA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Wataru MORIKAWA et al.
Serial No. 09/269,720 Group Art Unit: 1642
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7. Under my direction, in order to investigate relationship between an ability to bind to heparin (heparin affinity) and difference at the N-terminal sequence (Glu-LBS-I or Lys-LBS-I, more specifically N-terminal lysine-78, valine-79, tyrosine-80 or leucine-81), and relationship between an ability to bind to heparin (heparin affinity) and the presence or absence of glycosylation, the following experiments have been done.

Materials and Methods

(1) Isolation of Plasminogen and Its Related Fragments

Glu-plasminogen (hereinafter referred to as "Glu-plg.") was purified from pooled human plasma in the presence of 3 mM benzamidine-HCl, 5 mM EDTA, aprotinin (100 units/ml) and soybean trypsin inhibitor (10 μg/ml). The pooled plasma was first subjected to affinity chromatography on lysine-Sepharose 4B in 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA as described (Deutsch et al.[1]), followed by gel filtration on Sephacryl S-200.

Lys-plasminogen (hereinafter referred to as "Lys-plg.") was obtained by the plasmin auto-activation method. Briefly, the elute fraction obtained by the chromatography of Glu-plg. was concentrated (10 mg/ml) and then was dialyzed against 50 mM Tris-HCl, pH 6.5 overnight. To the concentrate was added 1 mM tranexamic acid and the mixture was incubated at 30°C overnight.

Angiostatin, including Kringles 1-3 of plasminogen, was purified from Glu-plg. or Lys-plg. according to the method of Sottrup-Jensen et al. (Sottrup-Jensen et al.[2]). The obtained angiostatins purified from Glu-plg. or Lys-plg. were referred to as "Glu-LBS-I" and "Lys-LBS-I" in Fig. 1, respectively.

[1]: Deutsch, D.G. and Mertz, E.T., Plasminogen: purification from human plasma by affinity chromatography, Science 170: 1995-1996, 1970

[2]: Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E., and Magnussen, S., The primary structure of human plasminogen; isolation of two lysine-binding fragments and "mini"-plasminogen (MW 38,000) by elastase-catalyzed-specific limited proteolysis, Progr. Chem. Fibrinolysis Thrombolysis., 3, 191-209, 1978, Raven Press, New York

(2) Analysis of N-terminal Amino Acid Residues and Carbohydrate Content

To Hitrap™ Heparin (1.0 ml) equilibrated with 10 mM Tris-Citrate (pH 6.0) containing 50 mM NaCl was applied each 1 ml

of Glu-LBS-I (1.0 mg/ml) and Lys-LBS-I (1.0 mg/ml) dissolved in the same buffer. The gel was washed with the buffer (10 ml) at a flow rate of 0.5 ml/min. Then, gradient elution was carried out with 50 mM NaCl/Tris-Citrate (10 ml), 1 M NaCl/Tris buffer (pH 7.2)(10 ml). Each of fractions with high, medium or low heparin affinity from Glu-LBS-I and Lys-LBS-I was applied on 12.5% SDS-PAGE, and stained with Coomassie Brilliant Blue.

The proteins separated by SDS-PAGE as bands A, B and C were electrophoretically transferred from the gels onto PVDF membranes. The protein bands on the membranes were stained with Coomassie Brilliant Blue R-250, excised, and subjected to analysis of N-terminal amino acid residues with an automatic protein/peptide sequencer (Applied Biosystems Model 477A). Carbohydrate compositions of the heparin affinity chromatography-resolved variants of Glu-LBS-I and Lys-LBS-I were assayed by the methods of Hayes et al. (Hayes et al.[3])

[3]: Hayes, M.L. and Castellino, F.J., Carbohydrate of the human plasminogen variants, J. Biol. Chem., 254, 8768-8771, 1979

Results

The results are shown in the attached Figure 1 and the following Tables 1 and 2.

Table 1. N-terminal amino acids of Glu-LBS-I and Lys-LBS-I

	Glu-LBS-I			Lys-LBS-I		
N-terminal amino acid residue	A[a]	B[a]	C[a]	A	B	C
Lys-78	-[b]	-	-	-	6.7[c]	8.5
Val-79	-	-	-	5.6	4.4	0.4
Tyr-80	8.0	10.1	13.0	1.5	2.0	0.3
Leu-81	1.2	1.5	1.6	-	-	-
Total	9.2	11.6	14.6	7.1	13.1	9.2

[a]: "A", "B" and "C" indicate heparin binding fractions shown in the attached Figure 1.
[b]: Not detectable (<0.1 pmol)
[c]: Contents of N-terminal amino acid in each band were represented as pmol.

Table 2. Analysis of carbohydrate contents of Glu-LBS-I and Lys-LBS-I

	Glu-LBS-I			Lys-LBS-I		
Carbohydrate residue	Low[(d)]	Medium[(d)]	High[(d)]	Low	Medium	High
Sialic acid	1.9	0.9	0.0	1.8	1.2	0.0
Fucose	0.0	0.0	0.0	0.0	0.0	0.0
N-Galactosamine	0.2	0.2	0.0	0.1	0.1	0.0
N-Glucosamine	0.8	0.0	0.0	0.7	0.3	0.0
Galactose	1.0	0.7	0.2	1.0	0.9	0.3
Glucose	0.0	0.1	0.0	0.3	0.1	0.0
Mannose	0.7	0.2	0.0	1.0	0.0	0.0
Total	4.6	2.1	0.2	4.9	2.6	0.3

(mol/mol of protein)

[(d)]: "Low", "Medium" and "High" indicate heparin binding fractions shown in the attached Figure 1.

The attached Figure 1 indicates three bands (A, B and C) on the SDS-PAGE gel separated from each of fractions with high, medium or low heparin affinity from Glu-LBS-I and Lys-LBS-I. Bands A, B and C correspond to K1-3 molecular species with MW 45, 40 and 38 kD, respectively.

It was found that as heparin affinity of the fractions becomes higher (from "low" to "high" heparin affinity), bands were shifted from A to C, i.e. from higher to lower MW.

This difference of MW in bands A to C reflects the presence or absence of glycosylation as shown in Table 2.

Table 1 investigates relationship between an ability to bind to heparin (heparin affinity) and difference at the N-terminal sequence (Glu-LBS-I or Lys-LBS-I, more specifically N-terminal lysine-78, valine-79, tyrosine-80 or leucine-81).

As shown in Table 1, the fraction with high heparin affinity from Lys-LBS-I, i.e. "C" in the column Lys-LBS-I, comprises predominantly Lys-LBS-I with N-terminal lysine-78 whereas Lys-LBS-I with N-terminal valine-79 or tyrosine-80 was present in a trace amount. On the other hand, Glu-LBS-I obtained from Glu-plg. through direct cleavage with elastase comprises predominantly Glu-LBS-I with N-terminal tyrosine-80 and in small portion with N-terminal leucine-81

irrespective of heparin affinity. It should be noted that Glu-LBS-I with N-terminal lysine-78 or valine-79 was utterly undetectable in fractions from Glu-LBS-I.

Table 2 investigates relationship between an ability to bind to heparin (heparin affinity) and the presence or absence of glycosylation.

As shown in Table 2, fractions with high heparin affinity from Lys-LBS-I or Glu-LBS-I comprise predominantly Lys-LBS-I or Glu-LBS-I without glycosylation, respectively. It appears that a degree of heparin affinity was conversely correlated with an extent of glycosylation.

In conclusion, the above results demonstrate that there exists correlation between an ability to bind to heparin (heparin affinity) and difference at the N-terminal sequence (Glu-LBS-I or Lys-LBS-I, more specifically N-terminal lysine-78, valine-79, tyrosine-80 or leucine-81).

The above results also demonstrate that there exists correlation between an ability to bind to heparin (heparin affinity) and the presence or absence of glycosylation.

The plasminogen fragment of the present invention has the activity to inhibit tumor metastasis and tumor growth but has substantially no activity to inhibit growth of endothelial cells of blood vessel. For this feature, the fragment must be Lys-LBS-I with N-terminal lysine-78 without glycosylation. As demonstrated in the above results, Lys-LBS-I with N-terminal lysine-78 as well as Lys-LBS-I or Glu-LBS-I without glycosylation is predominantly comprised in fragment with high heparin affinity. Thus, the plasminogen fragment of the present invention having the desired activity as mentioned above can be obtained by selecting high heparin binding fraction from elastase cleavage products of Lys-plg.

8. It is my opinion based on my knowledge and experience in this field that the fraction with high heparin affinity from Lys-LBS-I comprises predominantly Lys-LBS-I with N-terminal lysine-78 and without glycosylation. Thus, for obtaining the plasminogen fragment of the present invention having the activity to inhibit tumor metastasis and tumor growth but having substantially no activity to inhibit growth of endothelial cells of blood vessel, high heparin binding fraction from elastase cleavage products of Lys-plg. is selected.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-mentioned application or any patent issuing thereon.

This day of September, 2000.

Wataru MORIKAWA